J Vet Diagn Invest 18:443–447 (2006)

Determination of sheep prion gene polymorphisms from paraffin-embedded tissue

Robert A. Kunkle,[1] Janice M. Miller, David P. Alt, Randall C. Cutlip, Noelle E. Cockett, Shiquan Wang, Juergen A. Richt, Bruce V. Thomsen, S. Mark Hall

Abstract. Amino acid polymorphisms of the prion protein (PrP) greatly influence the susceptibility of sheep to scrapie. Selective breeding to increase the prevalence of PrP gene alleles associated with scrapie resistance is a flock management practice that is important for scrapie control programs. Determination of sheep PrP alleles typically has required extraction of DNA from host tissues that are freshly derived or stored frozen. We describe application of a DNA extraction procedure for formalin-fixed, paraffin-embedded tissues (PET) for the purpose of PCR amplification and nucleotide sequencing of relevant codons (136–171) of the sheep PrP gene. Tissues derived from 96 sheep were studied. The DNA sequence identity was confirmed in 87 of 94 matched samples of PET and frozen tissue specimens. DNA from brainstem PET of 2 sheep, from which fresh tissue was not available, was amplified and sequenced after formalin fixation for 7–70 days. This method will allow retrospective analysis of PrP genetics of sheep subsequent to postmortem diagnosis of scrapie when nonfixed tissue is unavailable for DNA extraction; however, it is not recommended that submission of fixed tissue supplant collection of fresh tissues for the purpose of determining PrP gene polymorphisms.

Key words: Gene sequencing; paraffin-embedded tissue; prion; scrapie; sheep.

Introduction

Scrapie is a contagious, uniformly fatal, neurodegenerative disease of adult sheep and goats. Definitive diagnosis is typically defined by postmortem pathologic changes, and detection of the disease-specific prion protein (PrP^{sc}) in affected tissues.[13] Most scrapie infections are presumably acquired by the oral route. Exposure to the scrapie agent initiates an autocatalytic conversion of the highly conserved, host-encoded, membrane-anchored, glycoprotein PrP^{c} to the abnormally folded PrP^{sc}. The clinical and pathologic hallmarks of scrapie develop as PrP^{sc} gradually accumulates in the central nervous system (CNS) months or years after exposure.

Immunohistochemical (IHC) detection of PrP^{sc} in lymphoid or CNS tissues of sheep is sufficient for the diagnosis of preclinical, as well as clinical scrapie. Commonly used IHC procedures utilize formalin-fixed, paraffin-embedded tissues (PET), as does routine histologic examination. In cases of clinical scrapie, histopathologic findings include neuronal vacuolar degeneration and loss, astrocytosis, and generalized microscopic vacuolation of neuropil in the CNS. Lesions are often inapparent in preclinical cases, for which diagnosis is achieved by detection of PrP^{sc}.

The susceptibility of sheep to scrapie is greatly influenced by host PrP gene alleles. Amino acid polymorphisms corresponding to the codons 136 and 171 are major determinants of relative susceptibility.[8,9] Residue 154 also has been found to influence scrapie susceptibility in a sheep breed.[4] The 5 common allelic variations, resulting from amino acid substitutions involving alanine (A), valine (V), arginine (R), histidine (H), and glutamine (Q), are $A_{136}R_{154}R_{171}$, $A_{136}R_{154}H_{171}$, $A_{136}H_{154}Q_{171}$, $A_{136}R_{154}Q_{171}$, and $V_{136}R_{154}Q_{171}$.[2] Nine other polymorphic sites (112, 127, 137, 138, 141, 143, 151, 176, and 211) also have been identified.[13] The V_{136} and Q_{171} haplotypes are linked to scrapie susceptibility, especially in the homozygous state.[4,8,9] Although the predictive value of PrP genotype in determining the eventuality of scrapie in an individual is not absolute, the VRQ/VRQ genotype is strongly associated with susceptibility, and the ARR/ARR genotype is strongly associated with resistance.[4,7] Therefore, sheep PrP gene polymorphisms found within a flock can de-

From the National Animal Disease Center, USDA, Agricultural Research Service, 2300 Dayton Avenue, PO Box 70, Ames, IA 50010 (Kunkle, Miller, Alt, Cutlip, Richt); and the Department of Animal, Dairy, and Veterinary Sciences, Utah State University, 4815 Old Main Hill, Logan, UT 84322-4815 (Cockett); and the US Sheep Experiment Station, ARS, USDA, H.C. 62, PO Box 2010, Dubois ID 83423-9602 (Wang); and the USDA, APHIS, VS, National Veterinary Services Laboratories, Ames, IA 50010 (Thomsen, Hall).

[1] Corresponding Author: Robert .A.. Kunkle, , DVM, , PhD, National Animal Disease Center, ARS, USDA, 2300 Dayton Avenue, PO Box 70, Ames, IA 50010. rkunkle@nadc.ars.usda.gov

termine recommendations for selective breeding to reduce or prevent scrapie occurrence.[3]

Determination of sheep PrP alleles typically has required extraction of DNA from host tissues such as blood, semen, buccal epithelial cells, brain, or potentially, almost all other tissues that are freshly derived or stored frozen .[9,15–19] These techniques are amenable to flock surveys, prospective studies, or retrospective analyses where fresh-frozen tissues are available. Retrospective studies to correlate PrP genetics and scrapie incidence can be problematic in instances where diagnosis is determined by IHC analysis and histologic examination but fresh tissues are not collected or frozen tissues are not available. The study reported here describes modifications of a previously published technique[11] for extraction of DNA from formalin-fixed, paraffin-embedded, sheep tissues that allow nucleotide sequencing of a segment of the PrP gene that encompasses codons 136 and 171.

Materials and methods

Sheep brainstem specimens collected from US sheep flocks were obtained from 2 sources. The first source was a group of 54 Suffolk, Hampshire, or cross-breed sheep collected from 3 scrapie-affected Midwestern US flocks and delivered to the National Animal Disease Center (NADC) in 1997. The sheep were euthanized by an overdose of IV administered sodium pentobarbital. Portions of lymphoid and CNS tissues (including the obex area of brainstem) were collected in buffered 10% formalin, and separate specimens of brainstem were frozen at −70°C. Brainstem specimens collected in formalin were kept in the fixative for a period of 2–8 days, and then were embedded in paraffin following routine histotechnologic methods. The second source of brainstem specimens was 40 PET blocks obtained from the National Veterinary Services Laboratories (NVSL) case submission archives. In addition to these 94 samples, portions of 2 additional sheep brainstem submissions to NVSL were collected and fixed in buffered 10% formalin for 7, 15, 31, or 70 days prior to processing into paraffin blocks. The PETs were sectioned and processed for DNA extraction, amplification, and sequencing.

Extraction of DNA from PET was based on a previously described method for crude extracts and purified preparations.[11] In brief, a crude tissue extract was obtained from 2 PET sections (each 5-μm thick) collected into a 1.5-ml microcentrifuge tube and pelleted by centrifugation at 16,000 × *g* for 1 min, followed by the addition of 200 μl of 0.05 M Tris (pH 7.5), 1 mM EDTA, and 0.5% Tween 20. The tube was then placed in a 100°C water bath for 10 min followed by snap-freezing (ethanol on dry ice); the 10-min boil and snap-freezing were repeated, followed by 1 more 10-min boil, and then were immediately centrifuged at 3,000 × *g* for 10 min to separate the paraffin while pelleting the tissue. Proteinase K[a] was added to the extract, via pipette tip through the resolidified paraffin overlay, to a final concentration of 0.5 U/ml (approx. 0.2 mg/ml) followed by incubation in a 42°C water bath for 16–20 hr. The inactivation of residual proteinase K was achieved by snap-freezing in cold ethanol and then a 10-min boil. The tissue debris was pelleted by centrifugation at 3,000 × *g* for 20 min. Supernatant was used either as crude extract or purified prior to amplification. Extracts were purified by removal of extraneous protein using a precipitating compound[b] followed by addition of LiCl (0.5 M final concentration) to the supernatant prior to ethanol precipitation of the DNA. The purified DNA preparation was pelleted by centrifugation, dried, and resuspended in 50 μl of a special water.[c] Target DNA amplification from either crude extract or purified preparation used a 3- to 20-μl volume of the suspension, equivalent to 0.5–1.0 μg of DNA, as determined by spectroscopic absorbance at 260 nm, in an amplification reaction mixture of a 100-μl total volume.

Each sample was amplified using a primer set,[d] producing a 304-nucleotide fragment encompassing codons 136–171 of the sheep PrP gene, designated as 4142 TGGAACAAGCCCAGTAAGCC and 9612 GGTGAAGTTCTCCCCTTGGT.[1] Other than the oligonucleotide primers used, amplification reaction mastermix was prepared as described.[11] The reaction mixture consisted of 80 μl of reaction mastermix and 20 μl of test sample. Amplification conditions were 5 min at 94°C for DNA polymerase activation and initial denaturation of target DNA; 50 cycles of 1 min at 94°C, 30 sec at 60°C, and 2 min at 72°C; and a final extension period of 10 min at 72°C. The amplification products were kept at 4°C for up to 18 hr prior to analysis by agarose gel electrophoresis, staining with ethidium bromide, and visualization with ultraviolet light. Amplified product was purified by use of a centrifugal filter device[e] prior to sequencing with oligonucleotides 4142 and 9612. Products were quantitated using the Pico Green assay for double stranded (ds)DNA.[f] The appropriate quantity of dsDNA PCR product was labeled in both directions using a terminator chemistry product[g] according to manufacturer's instructions. The labeled products were sequenced using a genetic analyzer.[h] Primers used for sequencing were identical to those used in the primary PCR reaction. Interpretation of sequence data was facilitated by use of special software.[i]

Determination of ovine PrP gene polymorphisms of codons 136 and 171 using freshly frozen brainstem obtained from the 54 sheep delivered to the NADC was performed as described.[17] The 40 sheep brainstem submissions to NVSL included fresh and formalin-fixed specimens; codon-136 and codon-171 amino acid polymorphism data obtained from fresh tissue (blood) was supplied from a commercial source.[j]

Results

Seven of the 94 cases examined failed to produce consistent sequencing results between DNA extracted from nonfixed tissue versus PET. The amino acid polymorphisms corresponding to codons 136 (A, V) and 171 (Q, R, H), obtained by nucleotide sequencing, were determined for all 94 freshly frozen tissues

or blood samples, and for 92 of the 94 PETs examined. In 2 cases, amplified product was not obtained from extracts of PET. The nucleotide sequences from the 92 positive PET samples were unambiguous; however, disparate results in codon sequences between PET and frozen tissue could not be resolved by retesting or reexamination of records in 2 cases. There were 3 additional cases in which the initial examination had indicated discrepancies between PET and frozen sample sequences. These disparate results were each resolved in different ways: 1) a clerical error discovered in the recorded interpretation of sequence data derived from frozen tissue was corrected, 2) retest of frozen tissue yielded sequence in agreement with PET sequence, and 3) although frozen and the initially tested PET brainstem specimen yielded dissimilar sequences, tests of all 5 additional PET from the same sheep yielded sequence data consistent with the frozen tissue findings. Therefore, in 87 of 94 cases, sequence data from PET was obtained that was in agreement with that derived from frozen tissue or blood on initial examination (Table 1). The 154-codon sequence was not determined for the fresh blood or frozen brainstem specimens; the 92 of 94 PET samples, from which amplified product was obtained, yielded sequence homozygous RR at codon 154 in all cases.

Sequence was obtained on first attempt from 72 of 94 PET crude extract amplification products. Amplification product was obtained and sequenced following a second attempt of crude extraction for 14 of 22 remaining PET samples. Thus, crude extract preparations did not yield amplification product for 8 of 94 PET samples; however, successful PCR amplification and generation of readable sequence from 6 of those 8 specimens were achieved once purified preparations were assayed. As previously mentioned, amplified product was not obtained from 2 of 94 PET samples despite repeated extractions.

Two sheep brainstem specimens were portioned and immersed in formalin for 7, 15, 31, or 70 days prior to processing in paraffin blocks; the duration of formalin fixation affected neither the ability to amplify target DNA nor to produce readable sequence of DNA amplicon.

Table 1. Sheep codon 136 and 171 polymorphisms obtained from frozen and paraffin-embedded (PET) tissue from the brainstem.

Frozen	PET	NADC (n = 54)	NVSL (n = 40)
AAQQ	AAQQ	26	11
AAQR	AAQR	15	13
AARR	AARR	3	5
AVQQ	AVQQ	4	4
AVQR	AVQR	0	5
VVQQ	VVQQ	0	1
Failures*		6	1

Amino acid substitutions: alanine (A), valine (V), arginine (R), histidine (H), and glutamine (Q). NADC = National Animal Disease Center; NVSL = National Veterinary Services Laboratories.

* Failures consist of inability to obtain polymerase chain reaction amplification product (n = 2) and sequence inconsistency between fresh or frozen tissues and PET (n = 5).

Discussion

The present method to extract, amplify, and sequence sheep DNA from PET, relevant to deducing the PrP gene amino acid polymorphisms associated with scrapie, produced results consistent with those obtained from freshly frozen brainstem in 87 of 94 cases examined. The disparate DNA sequence results obtained from fresh versus fixed tissue in 2 cases may indicate that noncomparable results reflect deficiencies in methodology, or the discrepancies may highlight the increased potential for clerical errors to occur when samples are split for independent analyses. In this study, 3 such labeling errors were apparently resolved after reexamination of records or tissues, or both. Since the amino acid polymorphisms obtained were of a common variety in both cases (AA_{136} versus AV_{136}, and QQ_{171} versus QR_{171}; Table 1), tissue labeling mistakes were suspected to a greater degree than the possibility of specimen contamination or technical error.

This PET DNA extraction method, principally consisting of repeated cycles of boiling and freezing, was originally applied to the PCR-based diagnosis of mycobacterioses,[10–12] diseases in which serologic diagnosis is confounded by cross-reactive dominant antigens, and isolation is delayed by the indolent growth characteristics of the bacteria. Application of the method to PrP genotyping for retrospective studies should be a simple undertaking for any institution currently using this particular technique for diagnosis of tuberculosis.

The majority (72/94) of PET samples examined were sequenced on the first attempt after amplification of crude DNA extracts, and a further 14 samples after a second attempt. These 14 initial failures may have more to do with variable amounts of tissue DNA in PET sections than the quality of DNA in the crude extract preparations since only 6 of 94 required preparations of purified DNA for amplification of sequence-ready product. The 2 of 94 failures to obtain PCR product from PET probably reflect inherent limitations of the method, as neither repeat extractions of serial sections nor purification of DNA extracts affected the outcome. Variables such as composition of fixative, storage conditions during

fixation, tissue dehydration conditions, quantity of sectioned tissue, or density of cell nuclei in the PET could affect recovery of suitable quality or quantity of DNA.

There was no apparent influence on the ability to amplify and sequence 2 sets of sheep brainstem PET when formalin fixation was extended from 7 to 70 days. However, as a general rule, the longer the fixation period, the more problematic amplification of extracted DNA becomes. Because of the degradation of nucleic acids in formalin-immersed tissues, it is recommended that primers for amplification of short DNA stretches (< 200 base pairs) be used for DNA isolated from fixed specimens.[6] In this study, use of the 4142/9612 primer set, which yields a small (304 bases) amplification product, may have limited the influence of formalin-induced DNA damage. A fixation period beyond 10 weeks would probably have lessened the efficiency of amplification, but that time frame was not established in this study.

The benefit of using PET as the source of DNA for prion genotype sequencing is relevant in instances where scrapie is diagnosed by histologic examination or IHC analysis, or both, and fresh tissues are not available. These instances arise when tissues are not collected appropriately or when freezer malfunction spoils stored frozen tissues.

As found in this study, mislabeled samples or recording errors could lead to discrepant results when tissue samples are divided for separate processing and analysis. Robust quality-control protocols are necessary to identify tissue handling mix-ups, and even when discovered, errors of this type typically are difficult to resolve. A variety of suspected clerical errors were uncovered in this study after some initial confusion over dissimilar sequence data obtained from 5 ''matched'' fixed and nonfixed samples; investigation of records and additional analysis apparently resolved 3 of these cases, but the discrepancies remain unresolved for 2 animals.

The stability of PET for the purpose of histologic examination is virtually unlimited, and institutionally maintained PET archives are common for this reason. Likewise, it can be reasonably assumed that consistent IHC results will be obtained from archived PET, though it may depend on the specific application. In the authors' experience, comparable PrP IHC results are obtained with PET sectioned years apart. The ''shelf-life'' of DNA in PET may be more limited. Duration of storage in paraffin block form for longer than 4 years has produced extracted DNA fragments of diminished size,[5] and decreased efficiency of PCR amplification has been reported for PET stored for 5 years or longer.[6] However, longer storage may not affect generation of small amplicons; successful amplification of 58- and 116-base pair fragments has been reported for DNA extracted from PET samples stored for 16 years.[14] In the study reported here, 54 of the PET had been embedded in paraffin 6–7 years prior to sectioning for DNA extraction, and amplified product was obtained from 52 specimens. The better-than-expected success rate may be attributed to the extraction method's minimalized steps for deparaffinization or recovery of template target DNA. Genotyping of relevant polymorphic sites from archived sheep PET would allow retrospective studies to augment our current understanding of the epidemiologic link between prion genetics and scrapie occurrence. Retrospective studies aside, submission of nonfixed tissues remains essential, as they are the most efficient method of DNA extraction for determination of sheep PrP gene polymorphisms.

Acknowledgements

The authors thank Dennis Orcutt, Martha Church, Jean Donald, Sharla Van Roekel, and Daniel Liebsch for technical assistance.

Disclaimer: Mention of trade names or commercial products in this report is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

Sources and manufacturers

a. AMRESCO Co., Solon, OH.
b. Procipitate, Ligochem, Fairfield, NJ.
c. Omnisolv, EM Science, Gibbstown, NJ.
d. Integrated Technologies, Coralville, IA.
e. Microcon YM-100, Amicon Corp., Bedford, MA.
f. Invitrogen Corporation, Carlsbad, CA.
g. Big Dye, Applied Biosystems Inc., Foster City, CA.
h. ABI 3100, Applied Biosystems Inc., Foster City, CA.
i. SeqMan II (version 5.08) Lasergene, DNASTAR, Inc., Madison, WI.
j. Gene Check, Fort Collins, CO.

References

1. Baylis M, Houston F, Goldmann W, et al.: 2000, The signature of scrapie: differences in the PrP genotype profile of scrapie-affected and scrapie-free UK sheep flocks. Proc R Soc Lond B 267:2029–2035.
2. Belt PBGM, Muileman IH, Schreuder BEC, et al.: 1995, Identification of five allelic variants of the sheep PrP gene and their association with natural scrapie. J Gen Virol 76:509–517.
3. Dawson M, Hoinville LJ, Hosie BD, Hunter N: 1998, Guidance on the use of PrP genotyping as an aid to the control of clinical scrapie. Vet Rec 142:623–625.
4. Diaz C, Vitezica ZG, Rupp R, et al.: 2005, Polygenic variation and transmission factors involved in the resistance/susceptibility to scrapie in a Romanov flock. J Gen Virol 86:849–857.
5. Goelz SE, Hamilton SR, Vogelstein B: 1985, Purification of DNA from formaldehyde fixed and paraffin embedded human tissue. Biochem Biophys Res Commun 130:118–126.

6. Greer CE, Peterson SL, Kiviat NB, Manos MM: 1991, PCR amplification from paraffin-embedded tissues. Effects of fixative and fixation time. Am J Clin Pathol 95:117–124.
7. Hunter N, Foster JD, Goldmann W, et al.: 1996, Natural scrapie in a closed flock of Cheviot sheep occurs only in specific PrP genotypes. Arch Virol 141:809–824.
8. Hunter N, Goldmann W, Foster JD, et al.: 1997, Natural scrapie and PrP genotype: case-control studies in British sheep. Vet Rec 141:137–140.
9. Hunter N, Moore L, Hosie BD, et al.: 1997, Association between natural scrapie and PrP genotype in a flock of Suffolk sheep in Scotland. Vet Rec 140:59–63.
10. Miller JM, Jenny AL, Ellingson JL: 1999, Polymerase chain reaction identification of *Mycobacterium avium* in formalin-fixed, paraffin-embedded animal tissues. J Vet Diagn Invest 11:436–440.
11. Miller JM, Jenny AL, Payeur JB: 2002, Polymerase chain reaction detection of *Mycobacterium tuberculosis* complex and *Mycobacterium avium* organisms in formalin-fixed tissues from culture-negative ruminants. Vet Microbiol 87:15–23.
12. Miller JM, Jenny A, Rhyan J, et al.: 1997, Detection of *Mycobacterium bovis* in formalin-fixed, paraffin-embedded tissues of cattle and elk by PCR amplification of an IS6110 sequence specific for *Mycobacterium tuberculosis* complex organisms. J Vet Diagn Invest 9:244–249.
13. Prusiner SB, Williams E, Laplanche JL, Shinagawa M: 2004, Scrapie, chronic wasting disease, and transmissible mink encephalopathy. *In:* Prion biology and diseases, ed. Prusiner SB, 2nd ed., pp. 545–594. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
14. Shuster DE, Kehrli ME, Ackermann MR, Gilbert RO: 1992, Identification and prevalence of a genetic defect that causes leukocyte adhesion deficiency in Holstein cattle. Proc Natl Acad Sci USA 89:9225–9229.
15. Townsend SJ, Warner R, Dawson M: 2005, PrP genotypes of rare breeds of sheep in Great Britain. Vet Rec 156:131–134.
16. Tuo W, O'Rourke KI, Zhuang D, et al.: 2002, Pregnancy status and fetal prion genetics determine PrP^{Sc} accumulation in placentomes of scrapie-infected sheep. Proc Natl Acad Sci USA 99:6310–6315.
17. Wang S, Cockett NE, Miller JM, et al.: 2002, Polymorphic distribution of the ovine prion protein (*PrP*) gene in scrapie-infected sheep flocks in which embryo transfer was used to circumvent the transmission of scrapie. Theriogenology 57: 1865–1875.
18. Yuzbusian-Gurkan V, Krehbiel JD, Cao Y, Venta PJ: 1999, Development and usefulness of new polymerase chain reaction-based tests for detection of different alleles at codons 136 and 171 of the ovine prion protein gene. Am J Vet Res 60:884–887.
19. Zhou H, Hickford GH, Fang Q: 2005, Technical note: determination of alleles of the ovine *PRNP* gene using PCR-single-strand conformational polymorphism analysis. J Anim Sci 83:745–749.